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1. Kim et al Cancer Research 1986 November Vol 46 (11) : 5985-92
2. Pour et al Int J Pancreatol. 1986 Dec vol 1 (5-6) : 327 - 40
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## Blood-group antigen expression during pancreatic cancer induction in hamsters

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(Received 14 February 1986; Accepted 20 May 1986)

Key words: Induced pancreatic tumors; blood group antigenicity; pancreas cancer

### Summary

The expression of blood group-related and tumor-associated antigens was examined in pancreatic adenocarcinomas and in the normal pancreas of hamsters to determine if this expression correlated with the host blood group and/or stage of carcinogenicity, respectively. Pancreatic tumors were induced by 4 weekly treatments of hamsters with *N*-nitrosobis(2-oxopropyl)amine (BOP) and analyzed immunohistochemically during different stages of tumor progression with polyclonal antibodies (PoAbs) and monoclonal antibodies (MoAbs) against A, B, O and Lewis (Le) isoantigens, including X, Y and CA 19-9 monosialoganglioside (gastrointestinal cancer antigen, GICA), as well as with PoAbs detecting human carcinoembryonic antigen (CEA),  $\alpha$ -fetoprotein (AFP) and the  $\beta$ -subunit of human chorionic gonadotropin ( $\beta$ -HCG). The red blood cells of both control and tumor-bearing hamsters expressed AB and Le<sup>(a+b+)</sup>-like blood group types, as detected by polyvalent antisera. However, none of the MoAbs reacted with the hamster red blood cells. In the pancreas, all PoAbs against blood group antigens reacted with hyperplastic ducts and ductules at very early stages of carcinogenesis, as well as with neoplastic lesions, but not with

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*Abbreviations:* CEA, carcinoembryonic antigen; AFP,  $\alpha$ -feto-protein;  $\beta$ -HCG, human choriogonadotropin  $\beta$ -subunit; BOP, *N*-nitroso-bis(2-oxopropyl)amine; GICA, gastrointestinal cancer antigen; IIP, indirect immunoperoxidase; MoAb, monoclonal antibody; PAP, immunoperoxidase-antiperoxidase; PBS, phosphate-buffered saline; PoAb, polyclonal antibody.

normal pancreatic cells, except for the acinar cells, which were stained with PoAb-B, PoAb-Le<sup>a</sup> and PoAb-Le<sup>b</sup>. None of the MoAbs showed any affinity for the normal pancreatic cells; however, they reacted to various degrees with induced hyperplastic and neoplastic tissue. Reactivities of several MoAbs with malignant cells were greater than those with hyperplastic lesions: MoAb-B was highly reactive with all induced lesions, MoAb-A less reactive, and MoAb-H and MoAb-Le<sup>a</sup> (which has 6 sugar chains) detected only some cancer cells. Neither of the two MoAb-Le<sup>b</sup> (with 5 carbohydrate chains) reacted with carcinoma cells, although they did bind to a few hyperplastic cells. Neither MoAb-Le<sup>a</sup> and MoAb CA 19-9, nor PoAbs against CEA, AFP and  $\beta$ -HCG, reacted with any normal, hyperplastic or malignant cells. These results demonstrate the differential reactivity of these PoAbs and MoAbs in normal and malignant pancreatic tissue and show that blood group antigens, especially the B isoantigens, are specific markers for induced pancreatic duct tumors in hamsters.

### Introduction

Numerous studies have demonstrated the expression of blood group ABO(H), Lewis<sup>a</sup> (Le<sup>a</sup>), Le<sup>b</sup>, I and T antigens by a variety of human neoplasms [1-27]. However, there are few studies of this type that deal specifically with pancreatic cancer [10-12]. We have recently shown that normal colon mucosa samples from colo-rectal carcinoma patients expressed Le<sup>a</sup>, but not Le<sup>b</sup>, antigens [4,12]. By contrast, Le<sup>b</sup> was expressed in all tumors of patients whose normal colon was Le<sup>b</sup>-negative, indicating that Le<sup>b</sup> is a colon adenocarcinoma-associated antigen [4]. In some human tumors [15,16,18,20,23] the expression of blood group antigens correlating with that of the host erythrocytes has been used as a marker for tumor diagnosis.

We have shown previously [28] that induced and transplanted pancreatic ductal/ductular adenocarcinomas in Syrian hamsters produce mucin that expresses antigenic determinants of a blood group A-like specificity. In the present study, experiments were done to determine: (1) whether induced pancreatic carcinoma cells in hamsters express antigens of other blood group types (B, Le<sup>a</sup>, Le<sup>b</sup>) and if so, whether the tumor antigens correspond to the host blood group antigens; (2) whether there are any differences in the binding patterns of antigens in induced lesions by employing PoAbs and MoAbs, and whether there is a correlation between morphological appearance and antigenic expression of tumors; (3) the stages of carcinogenesis at which the antigen(s) is expressed in amounts over that produced by normal tissue; and (4) whether induced pancreatic tumors express other antigens, such as CEA, AFP,  $\beta$ -HCG, and GICA, (19-9 monosialoganglioside), which are known to be expressed in human pancreatic cancer [25,26,29-34]. The reactivity of CA 19-9 was of particular interest because it is a sialylated Le<sup>a</sup>.

## Materials and Methods

### *Animals*

Eight-week-old outbred (Eppley colony) Syrian golden hamsters, weighing 110 g (males) and 115 g (females), were housed in plastic cages (Macrolon) on granular cellulose bedding (Bed-O-Cobs, Anderson Cob Co., Maumee, OH) in groups of 5 by sex. They were kept under standard laboratory conditions (temperature,  $22 \pm 3^\circ\text{C}$ ; relative humidity,  $40 \pm 5\%$ ; light/dark cycle, 12 h/12 h) and were given Wayne pelleted diet (Allied Mills, Chicago, IL) and water ad lib.

### *Chemicals and reagents*

BOP was synthesized by a method described earlier [35]. PoAbs and MoAbs against blood group substances were used to demonstrate blood group antigens in tissue. PoAb-A, PoAb-B, PoAb-Le<sup>a</sup>, and PoAb-Le<sup>b</sup> were purchased from American Dade, Miami, FL, and MoAb-A, MoAb-B and MoAb-H from DAKO Corporation, Santa Barbara, CA. MoAb detecting Le<sup>a</sup> (CO 514), MoAb-Le<sup>b</sup> (CO 432), MoAb-Le<sup>x</sup> (WGHS 29-1 and ZYG 111), MoAb-Le<sup>y</sup> (BR 55-2) and antibody from hybridoma 1116 NS-19-9 (CO 19-9) have been characterized by us [2-5,12,17,22,25,26,32-34,36,37]. PoAbs to human CEA, AFP and  $\beta$ -HCG were obtained from Immulok, Carpinteria, CA. Neuraminidase was from Sigma, St. Louis, MO, the DAKO PAP kit from DAKO Corporation, and normal goat serum and peroxidase-conjugated affinity-purified goat anti-mouse Ig G + M + A from Cappel Laboratories, Cochranville, PA.

### *Carcinogenesis study*

BOP, dissolved in physiological saline, was injected subcutaneously (10 mg/kg b.w.) into 18 female and 18 male hamsters weekly for 4 weeks, with no further treatment thereafter. Groups of these BOP-treated hamsters (3 females and 3 males) and an equivalent number of controls were killed 12, 16, 20, 27 and 30 weeks after the last BOP injection. Animals were completely necropsied and their pancreases were fixed in Bouin's solution for 24 h, dehydrated and embedded in paraplast by conventional methods and cut into serial sections. One of these sections was stained with hematoxylin and eosin, and the others were processed with PoAbs and MoAbs by the immunoperoxidase technique (see below).

### *Immunoperoxidase technique*

For MoAb-A, MoAb-B, MoAb-H, MoAb-Le<sup>a</sup>, MoAb-Le<sup>b</sup>, MoAb-Le<sup>x</sup>, MoAb-Le<sup>y</sup> and MoAb CA 19-9, the indirect immunoperoxidase technique was carried out

by a modification of the method of Primus and Goldenberg [38]. Deparaffinized sections were incubated with 0.3% hydrogen peroxide in absolute methanol for 30 min and rinsed with distilled water, followed by incubation with normal goat serum (diluted to 1:20 with PBS) for 10 min, and thereafter with primary MoAbs, each diluted 1:5 (for Le-related) or 1:50 (for ABH) in PBS for 2 h. After washing, tissues were incubated in peroxidase-conjugated affinity-purified goat anti-mouse Ig G + M + A (diluted 1:30 in PBS) for 1 h. Slides were then treated for 10 min with substrate containing 0.05% diaminobenzidine and 0.01%  $H_2O_2$  in 0.05 M Tris buffer, pH 7.6, counterstained with hematoxylin, dehydrated, and mounted in Permount.

The immunoperoxidase-antiperoxidase (PAP) technique was performed as described [39], using the DAKO PAP kit for PoAb-A, PoAb-B, PoAb-Le<sup>a</sup>, PoAb-Le<sup>b</sup>. Each primary antibody was diluted 1:25 in PBS. CEA, AFP and  $\beta$ -HCG were processed similarly, using the DAKO PAP kit, except that tissues were incubated with anti-CEA, anti-AFP and anti- $\beta$ -HCG, instead of PoAbs, for 20 min.

One group of sections was pretreated with 0.05 U of neuraminidase per slide for 1 h at room temperature prior to the application of MoAb CA 19-9, which has an antigenic determinant of sialylated-Le<sup>a</sup>.

Mouse myeloma P3 cell supernatant was used as a negative control for MoAbs. As a control for PoAbs, samples were incubated in PBS alone or with PoAbs preabsorbed on antigen-positive red blood cells. Positive controls were: human fetal liver (for AFP), colon tumors (for CEA) and placenta (for  $\beta$ -HCG).

#### *Blood group typing*

The blood group of each hamster was determined by the tube agglutination method using 2% hamster erythrocyte suspensions with PoAb-A, PoAb-B, PoAb-Le<sup>a</sup>, PoAb-Le<sup>b</sup>, MoAb-A, MoAb-B, and MoAb-H. A back typing was also performed in hamster serum using human type A, B and O reagent cells. Agglutination was checked microscopically.

#### *Histopathology*

Diagnostic criteria for induced lesions were as described previously [40,41]. In brief replacement of acinar cells by cells of a ductular character is termed 'pseudoductules'. The term Ca in situ refers to intraductal carcinoma or to focal malignant changes of ductular cells. Adenocarcinomas are characterized by invasive growth. Histology and terminology for the hamster ductal system have also been described [41].

#### *Method of histological evaluation*

In each slide the type and number of induced lesions were counted and their

reactivity with any of the antibodies was determined as a percentage of lesions homogeneously or heterogeneously stained (= % positively reactive lesions). In a given lesion, the percentage of positively stained cells was also estimated (= % reactive cells in a given lesion).

## Results

### *Incidence and types of induced lesions*

Hyperplasia of duct and ductular epithelium and formation of pseudoductules [41] were observed in hamsters killed 12 weeks after the last BOP injection. The intensity and multiplicity of these lesions increased, although not always in direct proportion to the time of killing. A ductular microcarcinoma was observed in a hamster at week 12, 2 ductular carcinomas in situ at weeks 16 and 20, respectively, 3 ductal/ductular adenocarcinomas at 27 weeks, 1 intraductal carcinoma at 33 weeks and 2 ductular adenocarcinomas at 37 weeks.

### *Blood group antigens in the normal hamster pancreas*

Table I lists the reactivity of PoAbs and MoAbs with the pancreatic cells of control hamsters. None of the PoAbs reacted with normal ductal, ductular and islet cells, whereas PoAb-B, PoAb-Le<sup>a</sup> and PoAb-Le<sup>b</sup> stained zymogen granules of acinar cells. If instead of these 3 PoAbs (containing both anti-IgG and IgM) human antisera (anti-IgG) was substituted, then the reactivity of acinar cell zymogens was significantly reduced.

None of the MoAbs reacted with normal pancreatic cells.

### *Tumor-associated antigens in the normal hamster pancreas*

CEA, AFP,  $\beta$ -HCG and GICA (CA 19-9) antigens were not demonstrated in any pancreatic cells.

### *Blood group antigens in induced pancreatic lesions*

The reactivity of PoAbs and MoAbs with induced pancreatic lesions is summarized in Table I. All four PoAbs reacted with all hyperplastic ductal and ductular cells, observed as early as 12 weeks (Figs. 1-4). The reactivity, in general, was independent of the type of PoAbs, although staining with PoAb-A and PoAb-B appeared to be more intense than that with PoAb-Le<sup>a</sup> and PoAb-Le<sup>b</sup>.

The reactivity of MoAbs with induced hyperplastic lesions, and in some cases also with neoplastic lesions, differed significantly from that of PoAbs, with the exception





Fig. 1. A hyperplastic major pancreatic duct in a hamster 12 weeks following BOP treatment. Hyperplastic ductal cells (middle and lower duct portion) show intense reaction on their luminal surface with PoAb-A, whereas normal ductal cells (upper ductal portion) are unstained. Peroxidase-antiperoxidase (PAP) technique.  $\times 150$ .

Fig. 2. Hyperplastic pancreatic duct during early stages of carcinogenesis (16 weeks following BOP treatment). Strong reaction of luminal cell portion of ductal cells and luminal content with PoAb-B. Islet (left) and acinar cells are unstained. PAP technique.  $\times 180$ .

of MoAb-B, for which the pattern of binding was similar to that of PoAb-A, i.e., it bound to all induced lesions, but not to any normal pancreatic cells (Fig. 5). Although all hyperplastic ductal and ductular cells were stained following MoAb-A, only a portion of the pseudoductular cells did so. Moreover, in a given pseudoductular focus, varying numbers of cells (1–100%) were stained. The binding levels of MoAb-H to hyperplastic ductal/ductular cells were about one-half those of MoAb-A, whereas the reactivity of these two antibodies with pseudoductular cells was similar. MoAb-Le<sup>a</sup> did not bind to any lesions, whereas 2–10% of hyperplastic ductal/ductular and pseudoductular structures were reactive with MoAb-Le<sup>b</sup> (up to 10% of the cells in a given lesion were positive).

MoAb-Le<sup>x</sup> bound to some cells of hyperplastic ductal/ductular and pseudoductular lesions, but not to cancer cells. MoAb-Le<sup>y</sup> reactivity was much better represented, and in any given hyperplastic duct/ductule and pseudoductule, the percentage



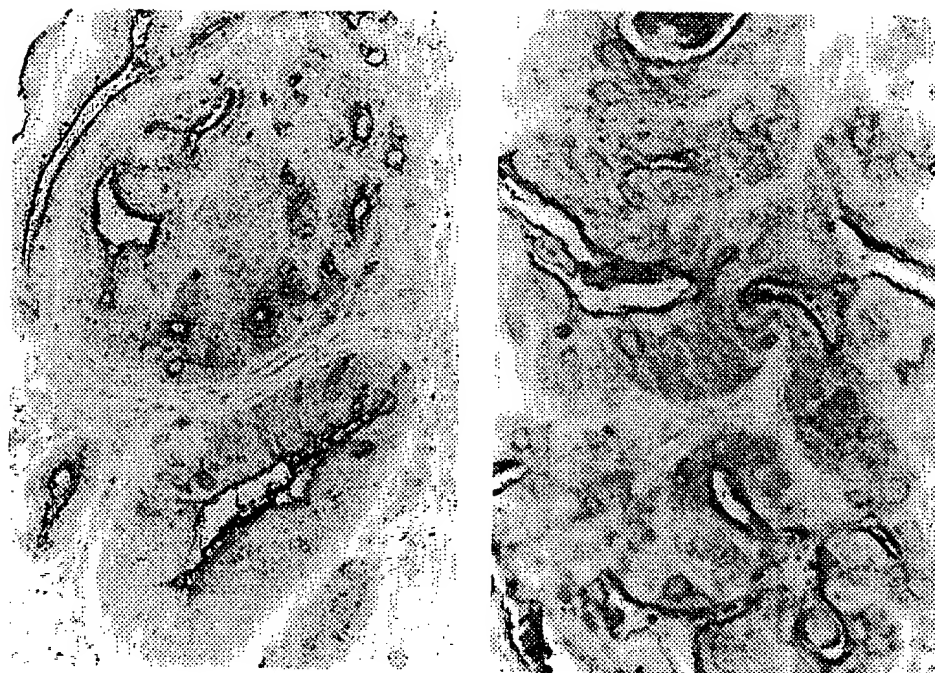


Fig. 3. Advanced ductal cell hyperplasia (at 27 weeks following BOP treatment) showing intense staining of the luminal cell portion with PoAb-B. In lower duct the stained material seems to have detached from cell surface. PAP technique.  $\times 150$ .

Fig. 4. A well-differentiated adenocarcinoma in a hamster at 33 weeks following BOP treatment. Surface of cancer cells is covered with thick layer of dark-stained material, partially shed into lumen. PoAb-A, PAP technique.  $\times 360$ .

of positive cells was considerably higher than that observed after staining with the two MoAb-Le<sup>a</sup>.

In contrast to the wide variation in the binding pattern of the antibody reagents with hyperplastic lesions, MoAb-A and MoAb-B reacted with all induced pancreatic ductal/ductular carcinomas in a pattern similar to that observed using the PoAbs (Fig. 6). However, the reactivity of MoAb-H, MoAb-Le<sup>b</sup> and MoAb-Le<sup>c</sup> with carcinoma cells was only partial, although all of these carcinomas were histologically well-differentiated, with focal cystic (papillary) structures (Figs. 6 and 7). Within a given carcinoma, the reactivity with MoAb-Le<sup>b</sup> and MoAb-Le<sup>c</sup> differed, i.e., each antibody bound to different cells.

The binding site for all of the PoAbs and MoAbs was primarily the luminal cell surface (glycocalyx pattern), and was only occasionally of a diffuse cytoplasmic nature. In adenocarcinomas, the antibodies were also strongly reactive with the cy-

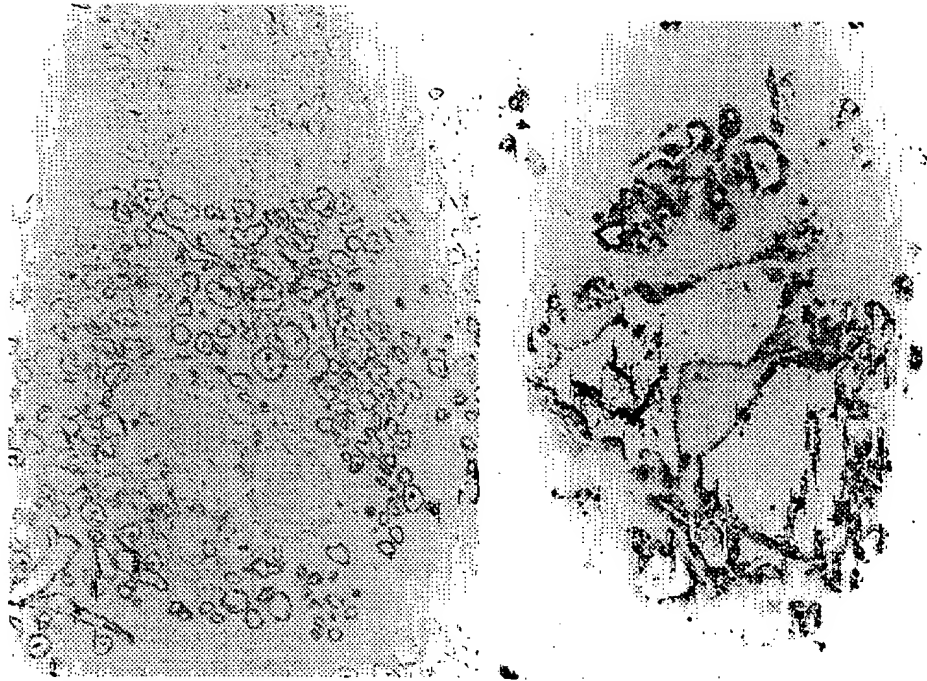


Fig. 5. Pseudoductular structure demonstrating strong staining of all cells with MoAb-B. In upper and lower portions distention of periinsular ductules is seen. Acinar cells (upper and right lower corners) and islet cells are unstained. Indirect immunoperoxidase (IIP) technique.  $\times 36$ .

Fig. 6. A section of adenocarcinoma showing expression of B antigen detected by MoAb-B. The intensity of the staining varies among the tumor cells, as does the localization of the antigen, most of which are in cell cytoplasm. Strong staining of luminal content. IIP technique.  $\times 72$ .

toplasm of basal cells. However MoAb-Le<sup>y</sup> stained the cells mostly in a granular pattern.

A strong positive reaction with PoAbs, as well as with MoAb-A and MoAb-B, was occasionally found in the periinsular region (Fig. 8), which apparently corresponded to the luminal content of periinsular ductules [41]. The erythrocytes and the surface of vascular endothelial cells within the pancreas were stained with PoAbs, but not with MoAbs. Macrophages were equally reactive with PoAbs, as with MoAb-A, MoAb-B, MoAb-H, MoAb-Le<sup>b</sup> and MoAb-Le<sup>y</sup>.

#### *CEA, AFP, $\beta$ -HCG and CA 19-9 antigen in induced lesions*

None of these antibodies stained any of the induced lesions. MoAb CA 19-9 in specimens treated with or without neuraminidase did not bind to any pancreatic cells, except to a few macrophages, which were also similarly reactive with MoAb-Le<sup>b</sup>.

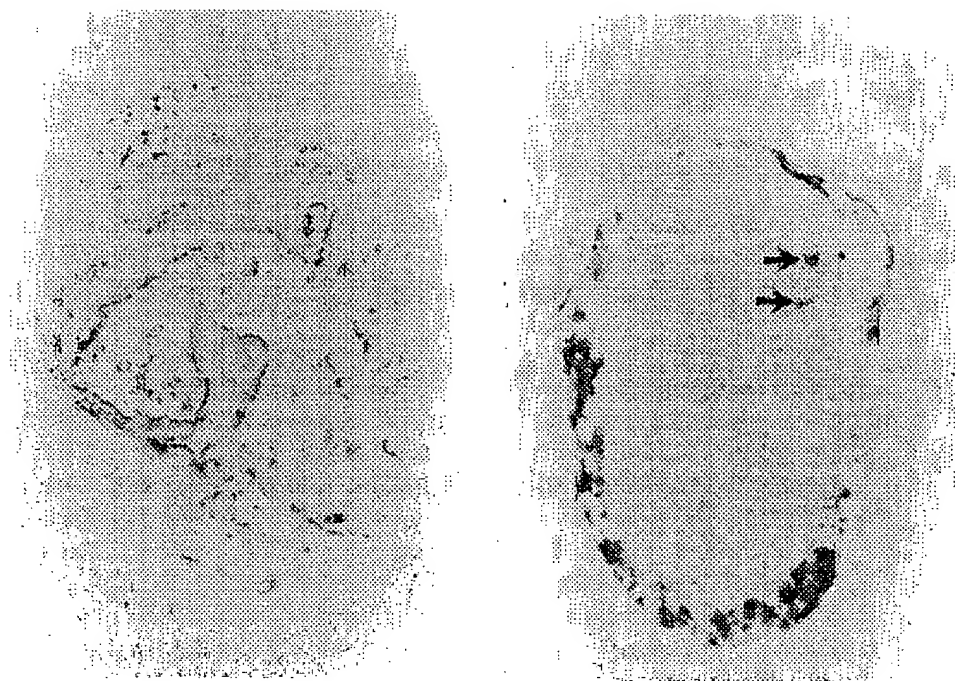


Fig. 7. The same tumor as in Fig. 6 treated with MoAb-H. The localization of the antigen is confined to the cell surface of most, but not all tumor cells. Weaker staining of the luminal content compared to that seen in Fig. 6. IIP technique.  $\times 72$ .

Fig. 8. Expression of anti-A in periinsular and intrainsular areas (arrows). Normal ductal (upper right corner), acinar and islet cells are unstained. MoAb-A, IIP technique.  $\times 210$ .

#### *Control slides*

All of the corresponding control tissues processed by this technique remained unstained.

#### *Blood group type of hamsters*

The erythrocytes from all BOP-treated hamsters and from 36 controls were agglutinated with the 4 polyvalent human anti-A, anti-B, anti-Le<sup>a</sup> and anti-Le<sup>b</sup> sera (with a 2+ score for both A and B antigens and a 1+ score for either Le<sup>a</sup> or Le<sup>b</sup> antigens). No reaction was observed between the hamster sera and A, B and O human blood cells. Consequently, all hamsters were considered to be group AB Le<sup>(a+b+)</sup> like.

None of the MoAbs reacted with the hamster red blood cells.

TABLE I  
THE REACTIVITIES OF POLYCLONAL ANTIBODIES AND MONOCLONAL ANTIBODIES TO NORMAL PANCREATIC TISSUE AND TO INDUCED PANCREATIC LESIONS

Antibody	Normal pancreas			Induced pancreatic lesions				
	Acinar cells	Ductal/ductular cells	Islet cells	Reactivity <sup>a</sup> (%)	Pseudoductules <sup>b</sup> (%)	Hyperplastic ducts/ductules <sup>b</sup> (%)	Carcinoma <sup>b</sup> (%)	
PoAb-A	0	0	0	100	100	100	100	
PoAb-B	+	0	0	100	100	100	100	
PoAb-Le <sup>a</sup>	+	0	0	100	100	100	100	
PoAb-Le <sup>b</sup>	+	0	0	100	100	100	100	
MoAb-A	0	0	0	5-80	1-100	100	100	
MoAb-B	0	0	0	100	100	100	100	
MoAb-H	0	0	0	0-40	2-100	10-50	1-100	
MoAb-Le <sup>a</sup>	0	0	0	0	0	0	0	
MoAb-Le <sup>b</sup>	0	0	0	2-10	0-10	0-10	1-100	
MoAb-Le <sup>c</sup>								
(WGHs 29-1)	0	0	0	0-1	0-50	0-60	0	
(ZYG 111)	0	0	0	0-1	0	0-1	0	
MoAb-Le <sup>d</sup>								
(BR 55-2)	0	0	0	5-30	5-100	0-3	6-100	

<sup>a</sup> Refers to the percent of the induced lesion stained homogeneously or heterogeneously in a given section.

<sup>b</sup> Percentage of stained cells in a given lesion.

## Discussion

In the present study, A-like, B-like, Le<sup>a</sup>-like and Le<sup>b</sup>-like antigens were detected in hamster erythrocytes by the four polyvalent human blood group antisera, but not by any of the respective MoAbs. Unlike human adults, hamster erythrocytes are both Le<sup>a</sup>- and Le<sup>b</sup>-positive, when reacted with polyvalent anti-Le sera. The Le<sup>(a+b+)</sup> phenotype is occasionally encountered in infants and young children, who subsequently become Le<sup>(a-b+)</sup> [42]. Although blood group substances of animal origin have been thought to belong to the same family as those in humans [24], it is possible that hamsters have a unique blood group substance that crossreacts with antigenic specificities present in all polyclonal antisera tested.

Normal hamster pancreatic parenchymal cells did not express any of the blood group antigens, when tested with PoAbs, except for acinar cells, which reacted with PoAb-B, PoAb-Le<sup>a</sup> and PoAb-Le<sup>b</sup>. A similar pattern of reactivity in acinar cells was observed, using *Griffonia simplicifolia*-I, a lectin with anti-B activity and with a specific affinity for  $\alpha$ -D-Gal and  $\alpha$ -D-GalNAc [43]. Whether these carbohydrate moieties determine the binding of PoAbs to acinar cells remains to be clarified, as none of the respective MoAbs showed a similar specificity for these cells. The absence of MoAb binding to normal ductal and ductular cells is consistent with our previous study in which selected lectins with blood group specificity were tested on the pancreas of untreated and BOP-treated hamsters [43].

Induced pancreatic ductal/ductular lesions, in contrast to the normal structures, reacted with all but one (MoAb-Le<sup>a</sup>) of the blood group antibodies, although reactivity varied greatly among individual antibodies. All four PoAbs showed binding to every induced lesion, the PoAb-A being the more specific. However, among the MoAbs, only MoAb-B bound to all lesions, whereas the binding of MoAb-A was inconsistent. Together with the great variation in the binding patterns of MoAb-Le<sup>a</sup> and MoAb-Le<sup>b</sup> with different carbohydrate specificities, these observations suggest that subtle structural changes can alter binding ability.

The quantitative differences in binding patterns of the antibodies to hyperplastic lesions, but their consistent affinity for malignant cells, are in accord with our previous study with selected lectins [43]. Thus, it is possible that cancerous cells produce substances recognized by each of these antibodies or that the synthesis of hamster-specific oligosaccharides is incomplete. However, the binding of MoAb-Le<sup>a</sup> to hyperplastic, but not to malignant cells, and the great variability in binding of MoAb-H, MoAb-Le<sup>b</sup> and MoAb-Le<sup>a</sup> to adenocarcinomas, indicate the involvement of several factors in antigen-antibody interactions in benign and malignant cellular changes.

There seem to be differences between human and hamster pancreatic tissues relative to the expression of ABH and Le isoantigens. Le antigens are found in normal human pancreatic cells: Le<sup>a</sup> has been identified in acinar, terminal ductular, and some ductal cells, and Le<sup>b</sup> in acinar and ductal epithelium [12]. However, these antigens cannot be demonstrated in untreated hamsters using the same MoAbs. The binding



of PoAbs-Le to hamster acinar cells could well represent non-specific crossreactivity.

Contrary to human carcinoma cells, which express both Le<sup>a</sup> and Le<sup>b</sup> [12], hamster pancreatic cancer cells do not react with MoAb-Le<sup>a</sup>. On the other hand in hamsters, all of the ABH isoantigens are expressed in cancerous cells, whereas human pancreatic cancers have been found either to express A or B antigen corresponding to the individual blood group types when MoAb was used [12], or to lose blood group antigenic expression in 80% of the cases, when mixed cell agglutination reaction was employed [10].

Hamster pancreatic cancer cells, unlike their human counterparts, also do not express GICA (CA 19-9), CEA, AFP or  $\beta$ -HCG antigens, although an oncofetal antigen-like activity has been demonstrated in the serum of pancreatic cancer-bearing hamsters [44] and in cell colonies established from a transplantable hamster pancreatic cancer [45]. The negative reaction of MoAb CA 19-9 with induced pancreatic lesions could be due to the lack of Le<sup>a</sup> antigen in hamster tissue. A similar negative reaction of CA 19-9 with human pancreatic cancer cells has been found in Le<sup>(a-b-)</sup> persons [22].

The detection of blood group antigens in induced lesions, but not in normal pancreatic structures, suggests that the expression of these antigens, especially the B isoantigens in hamsters, is tumor-specific, unlike the situation in man. Such antigen expression might result from newly synthesized glycoproteins in altered cells, precursor oligosaccharide accumulation, or increased synthesis of otherwise cryptic and immunologically undetectable antigens in normal cells [16].

#### Acknowledgements

This work was supported by Public Health Service grant R26 CA20198-06 1 from the National Pancreatic Cancer Project, Division of Cancer Cause and Prevention, National Cancer Institute and by NIH grants CA-10815 and CA-21124. The excellent technical help of Kathy Stepan, the editorial assistance of Marina Hoffman and Mardelle Susman and the secretarial assistance of Liz Stone are greatly appreciated.

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